1 **A DNA vaccine for Crimean-Congo hemorrhagic fever protects against disease and death**
2 **in two lethal mouse models**

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9 **Running title: Crimean-Congo hemorrhagic fever DNA vaccine**

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32   **Abstract**

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34   Crimean-Congo hemorrhagic fever virus (CCHFV) is a tick-borne virus capable of causing a

35   severe hemorrhagic fever disease in humans. There are currently no licensed vaccines to prevent

36   CCHFV infection. We developed a DNA vaccine expressing the M-segment glycoprotein genes

37   of CCHFV and assessed its' immunogenicity and protective efficacy in two lethal mouse models

38   of disease: type I interferon receptor knockout (IFNAR$^{-/-}$) mice; and a novel transiently immune

39   suppressed (IS) mouse model. Vaccination of mice by muscle electroporation of the M-segment

40   DNA vaccine elicited strong antigen-specific humoral immune responses with neutralizing titers

41   after three vaccinations in both IFNAR$^{-/-}$ and IS mouse models. To compare the protective

42   efficacy of the vaccine in the two models, groups of vaccinated mice (7-10 per group) were

43   intraperitoneally (IP) challenged with a lethal dose of CCHFV strain IbAr 10200. Weight loss

44   was markedly reduced in CCHFV DNA-vaccinated mice as compared to controls. Furthermore,

45   whereas all vector-control vaccinated mice succumbed to disease by day 5, the DNA vaccine

46   protected >60% of the animals from lethal disease. Mice from both models developed

47   comparable levels of antibodies, but the IS mice had a more balanced Th1/Th2 response to

48   vaccination. There were no statistical differences in the protective efficacies of the vaccine in the

49   two models. Our results provide the first comparison of these two mouse models for assessing a

50   vaccine against CCHFV and offer supportive data indicating that a DNA vaccine expressing the

51   glycoprotein genes of CCHFV elicits protective immunity against CCHFV.

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53   **Author summary**

54   Crimean-Congo Hemorrhagic Fever Virus (CCHFV) is a tick-borne virus capable of causing

55   lethal human disease against which there are currently no approved vaccines. In this study, we

56   compared the immunogenicity and protective efficacy of a candidate DNA vaccine expressing

57   the glycoprotein precursor gene of CCHFV in two mouse models. In addition to the recently

58   established IFNAR$^{-/-}$ mouse pathogenesis model, we also tested the vaccine in a novel murine

59   system in which the interferon (IFN) α/β signaling response of immunocompetent mice is

60   transiently suppressed. We found that the DNA vaccine elicited high humoral immune responses

61   and provided significant protection against challenge with CCHFV in both mouse models. These

62   findings further our understanding of the requirements for a CCHFV vaccine and provide a new

63   mouse model for the development of CCHFV vaccines.

64

Introduction

66   Crimean-Congo hemorrhagic fever virus (CCHFV) is a tick-borne virus with a wide

67   geographical distribution, including Africa, the Balkans, the Middle East, Russia and western

68   Asia (1). CCHFV, a member of the *Nairoviridae* family in the *Bunyavirales* order, has a

69   tripartite, negative-sense RNA genome comprising small (S), medium (M) and large (L)

70   segments. The small segment encodes the nucleocapsid protein (N), the M segment encodes the

71   glycoprotein open reading frame (ORF) that is cleaved into two structural glycoproteins (G$_N$ and

72   G$_C$) and non-structural proteins, and the L segment encodes the RNA-dependent RNA

73   polymerase (reviewed in (2)). CCHFV infection can cause Crimean-Congo hemorrhagic fever

74   (CCHF), a severe, often fatal, human disease characterized by hyperthermia and hemorrhage.

75   Humans appear to be uniquely affected by CCHFV as infection in other animals, including

76   agricultural animals, does not cause significant disease and the virus is generally cleared after a

77   brief period of viremia (3, reviewed in (4)). Human infection can result from the bite of infected

78   ticks, as well as from exposure to infected agricultural animals during butchering (5).

79   Nosocomial CCHFV infections primarily impacting medical staff have also been reported (6, 7).

80   Between 1953 and 2010, around 6000 cases of CCHF were reported worldwide with mortality

81   rates ranging from 5-67%, and from 2002 to 2016 more than 9700 CCHF patients were reported

82   in Turkey alone (5, 8, 9). There is also some evidence that the range of CCHFV is expanding, as

83   CCHFV infected ticks were found in Spain in 2010 and the first reported human infections in

84   Southwestern Europe occurred in Spain in 2016 (10, 11). As of 2017, CCHFV has been

85   designated as one of ten priority emerging infectious diseases by the World Health Organization.

86   This has led to an increased awareness of the need for medical countermeasures aimed at

87   preventing this disease.

88   To date, the only CCHFV vaccine tested in humans is a formalin inactivated, suckling

89   mouse brain-derived, virus preparation formulated with an aluminum hydroxide adjuvant, which

90   was developed in Bulgaria (12). Evaluation of this vaccine in healthy human volunteers showed

91   that four vaccinations elicited high levels of total IgG but only low levels of neutralizing

92   antibodies (13). Individuals vaccinated four times were also found to have T-cell responses to N

93   that were approximately ten-fold higher than those individuals receiving a single vaccination.

94   The historical absence of a lethal animal model of CCHF has precluded laboratory evaluation of

95   the efficacy of this vaccine, and controlled human studies have not been reported.

96   Although CCHFV is apathogenic in wild-type mice, two lethal mouse models, a STAT-1

97   knockout mouse model and an interferon α/β receptor 1 (IFN-α/β) knockout (IFNAR$^{-/-}$) mouse

98   model, have been developed that recapitulate the clinical features of CCHF in humans, including

99   severe hepatic injury (14-16). Both of these mouse systems have been used to evaluate CCHFV

100   vaccines. A study in the STAT-1 mouse model showed that a CCHFV subunit vaccine could

101   elicit strong neutralizing antibodies; however, the mice were not protected from lethal disease,

102   indicating that the STAT-1 model, which has defects in both type I and II interferon signaling

103   systems, is perhaps too sensitive to CCHFV for vaccine evaluation (17, 18). In contrast,

104   experimental CCHFV vaccines have recently been reported to show protective efficacy in the

105   IFNAR^{-/-} (A129) model (19, 20). This includes a formalin inactivated CCHFV (cell culture

106   derived Turkey-Kelkit06) vaccine that demonstrated protective efficacy in IFNAR^{-/-} (A129)

107   against a lethal infection with the homologous strain of CCHFV. Additionally, a modified

108   vaccinia Ankara (MVA)-vectored vaccine expressing the CCHFV M- segment ORF (MVA-GP),

109   from the IbAr 10200 strain, provided complete protection from lethal infection with the

110   homologous strain of CCHFV (19, 21). Investigation of vaccine-induced immune responses with

111   the MVA-GP vaccine suggested that both the cellular and humoral arms were critical for

112   protective efficacy. A CCHFV DNA vaccine comprised of three separate plasmids encoding G_N,

113   G_C, and N, tethered to a ubiquitin coding sequence, was also shown to elicit protective immunity

114   in IFNAR^{-/-} A129 mice (22).

115   We previously developed a CCHFV DNA vaccine encoding the CCHFV M-segment

116   ORF (23). We showed that this vaccine induced neutralizing antibodies in mice when delivered

117   by gene gun, but that the response was inconsistent. Efficacy testing was not possible at the time

118   due to the lack of a lethal animal model for CCHFV. Here, we report the improvement of this

119   DNA vaccine by gene optimization of the full length M segment. We evaluated the

120   immunogenicity and protective efficacy of this optimized vaccine when delivered by

121   intramuscular electroporation (IM-EP) in two lethal CCHFV models, IFNAR^{-/-} mice and a novel

122   transiently immune-suppressed (IS) C57BL/6 mouse CCHFV model (Golden , J.W., unpublished

123   data). The IS mouse model exploits a monoclonal antibody (MAb-5A3) that blocks signaling via

124   the IFNAR-1 subunit of the murine IFN α/β receptor. This transient IFN blockade has been used

125   in several other viral studies to examine the role of type I IFN in disease (24-26). The advantage

126   of the transient IFN-α/β blockade model is that vaccines can be evaluated in mice with intact

127   IFN-α/β signaling, and then during challenge IFN- α/β can be blocked to test protective efficacy.

128   To our knowledge, this is the first direct comparison of the IFNAR^{-/-} and IS mouse model for

129   assessing the immunogenicity and efficacy of a CCHFV vaccine.

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Materials and methods

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Ethics Statement

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133   This work was supported by an approved by the USAMRIID IACUC animal research protocol.

134   Research was conducted under an IACUC approved protocol in compliance with the Animal

135   Welfare Act, PHS Policy, and other Federal statutes and regulations relating to animals and

136   experiments involving animals. The facility where this research was conducted is accredited by

137   the Association for Assessment and Accreditation of Laboratory Animal Care, International and

138   adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, National

139   Research Council, 2011 (27). This research was conducted at a facility that is accredited by the

140   Association for Assessment and Accreditation of Laboratory Animal Care International

141   (AAALAC). Humane endpoints were used during these studies, and mice that were moribund,

142   according to an endpoint score sheet, were humanely euthanized. Mice were euthanized by CO_2

143   exposure using compressed CO_2 gas followed by cervical dislocation. However, even with

144   multiple observations per day, some animals died as a direct result of the infection.

145   **Cells and virus**

TR-17-120

Distribution Statement A: Approved for public release; distribution is unlimited.

146   HepG2 cells were propagated in Modified Eagles Medium with Earle's Salts (MEM) (Corning)

147   supplemented with 10% fetal bovine serum (FBS) (Gibco), and 1X Glutamax (Gibco). BHK-21

148   cells were cultured in Dulbecco's modified eagles medium (DMEM) (Corning) supplemented

149   with: 10% heat-inactivated FBS, 1% Penicillin/Streptomycin (Gibco), 1% Sodium Pyruvate

150   (Sigma), and 1% L-Glutamine (HyClone). SW13 cells were cultured in DMEM supplemented

151   with 10% FBS, 1% Penicillin/Streptomycin, 1% HEPES (Sigma), 1% non-essential amino acids

152   (Gibco), and 1% L-Glutamine. COS-7 cells were propagated in MEM supplemented with 10%

153   heat-inactivated FBS, 1% Penicillin/Streptomycin, and 1% L-Glutamine. All cells were

154   maintained at 37°C/5% CO_2. CCHFV strain IbAr10200 (USAMRIID collection) was used for all

155   experiments. This virus was previously passaged nine times in suckling mouse brain and then

156   propagated three times in HepG2 cells. The virus was collected from clarified cell culture

157   supernatants and stored at -80°C. All CCHFV work was handled in BSL-4 containment.

158   **DNA vaccine construction**

159   The M-segment ORF of strain IbAr10200 (Accession # AAA86616) was optimized by GeneArt

160   for human codon usage and deletion of known motifs that are detrimental to mRNA stability or

161   expression. The optimized gene was *de novo* synthesized and cloned into pCAGGS (a generous

162   gift from Robert Doms, University of Pennsylvania). The codon-optimized M-segment was

163   subcloned into the mammalian expression vector pWRG7077 at the NotI sites to create the

164   CCHFV-M DNA vaccine (23, 28). Nucleotide sequences were determined prior to vaccination.

165   **Flow cytometry**

166   COS-7 cells were propagated in 12-well tissue culture plates (Corning) to 70-90% confluency in

167   MEM. The medium in these plates was replaced with an Opti-MEM (Gibco) solution containing

168   2% FBS and 0.1% Gentamicin (Sigma) (cOpti-MEM). The DNA plasmids were transfected into

169   COS-7 cells in a dilution series of 500, 250, 100, 50, or 0 ng in triplicate using Fugene 6

170   (Promega) according to manufacturer's directions. Transfected cells were incubated for 44 h,

171   washed once with PBS and then detached by adding 100 µL of trypsin-EDTA (Gibco) per well

172   and incubating at ambient temperature. The cells were washed three times in FACS buffer

173   solution, PBS with 2% heat-inactivated FBS and 0.1% sodium azide (Sigma), fixed in Cytofix

174   buffer (BD Biosciences) for 30 minutes at 4°C, and then washed once with FACS buffer as

175   above. To detect the intracellular CCHFV glycoprotein, cells were permeabilized with

176   Perm/Wash buffer (BD Biosciences) at ambient temperature for 15 minutes, and then centrifuged

177   for five minutes at 980 x g, at 4°C. The permeabilized and non-permeabilized cells were

178   incubated with 20 µg/ml of anti-CCHFV G_C mouse monoclonal antibody 11E7 (USAMRIID) in

179   Perm/Wash buffer or PBS with 2% FBS respectively, and incubated for 30 minutes at 4°C. Cells

180   were washed three times in Perm/Wash buffer and centrifuged for five minutes at 980 x g, at 4°C

181   between each wash. Alexa Fluor 488-conjugated goat anti-rabbit IgG (Life Technologies) was

182   diluted 1:200 and incubated with the cells for 20 minutes at 4°C, and then washed three times

183   with FACS buffer. Cell pellets were re-suspended in 500 µl FACS buffer and analyzed on a

184   FACSCalibur flow cytometer (BD Biosciences). Cells staining positive for intracellular

185   glycoprotein were shown as a percentage of total cells per 10,000 events. Histograms and dot

186   plots were generated using FlowJo flow cytometry analysis software (Tree Star Inc).

187   **Western blot**

188   Following the aforementioned 44 h incubation post-transfection, COS-7 cells were lysed with 1X

189   Protein Loading Buffer (LI-COR). The cell lysates were probe sonicated for 15-20 seconds each,

190   mixed 9:1 with 2-mercaptoethanol (Sigma), and heated at 70°C for 10 min. Proteins were

191   separated by SDS-PAGE in 10% Bis-Tris gels (Nu-PAGE) and transferred to polyvinylidene

192   difluoride membranes (Invitrogen). The membranes were blocked with Odyssey Blocking Buffer

193   in Tris–buffered Saline (TBS, LI-COR) and probed for either G_C with 4.1 µg/ml of monoclonal

194   antibody 11E7 (USAMRIID) or G_N with 1:500 rabbit polyclonal anti-CCHFV G_N sera (a

195   generous gift from Robert Doms, University of Pennsylvania) (29) prepared in TBS (Sigma)

196   supplemented with 0.2% Tween-20 (TBST, Sigma) and incubated at 4°C overnight. The

197   membranes were washed 3 times with TBST and incubated with IR680-conjugated anti-rabbit or

198   IR800-conjugated anti-mouse secondary antibodies (LI-COR) diluted in TBST at ambient

199   temperature for 1 hour. The membranes were washed an additional 3 times with TBST and

200   imaged using an Odyssey CLx imaging system (LI-COR).

201   **DNA vaccination**

202   Groups of 10 IFNAR$^{-/-}$ or C57BL/6 mice were vaccinated in the anterior tibialis muscle with 25

203   µg of either the CCHFV-M co-DNA vaccine (IbAr10200) or the pWRG7077 empty vector using

204   the Ichor TriGrid® IM-EP system (30), under isoflurane anesthesia. All mice were vaccinated

205   three times at three weeks intervals. Blood was obtained via submandibular bleeds prior to each

206   vaccination.

207   **CCHF VLP production**

208   Production of IbAr10200 strain of CCHF VLPs (CCHF$_{VLP}$) was performed with slight

209   modification of methods reported previously (31). Briefly, BHK-21 cells were propagated to 70-

210   80% confluency in 10 cm^2 round tissue culture plates and then transfected with 10 µg pC-M Opt

211   (IbAr10200), 4 µg PC-N, 2 µg L-Opt, 4 µg T7-Opt, and 1 µg Nano-luciferase encoding mini-

212   genome plasmid using the Transit LT-1 (Mirus Bio) transfection reagent according to

213   manufacturer's instructions. Three days post-transfection, supernatants were harvested, cleared

214   of debris, and VLPs were pelleted through a cushion of 20% sucrose in virus resuspension buffer

215   (VRB; 130 mM NaCl, 20 mM HEPES, pH 7.4) by centrifugation for 2 h at 106,750 x g in an

216   SW32 rotor at 4℃. VLPs were resuspended overnight in 1/200 volume VRB at 4°C, and then

217   frozen at -80°C in single-use aliquots. Individual lots of $CCHF_{VLP}$ were standardized by Western

218   Blot analysis based on incorporation of N relative to a parallel gradient of recombinant N loaded

219   on the same SDS-PAGE reducing gel. $CCHF_{VLP}$ were also quantified using a TCID50 assay on

220   SW13 cells in 96-well, black-walled, clear-bottom plates (Corning). Plates were incubated with

221   tenfold dilutions of the $CCHF_{VLP}$ overnight and were then processed for Nano Luciferase

222   (Promega) expression. Wells that displayed a Nano Luciferase signal 3 standard deviations or

223   greater above background levels were considered positive for VLP signal. VLP stock

224   concentrations ($TCID_{50}$ per mL) were calculated using the Reed and Muench formula (32).

225   **CCHF VLP neutralization assay**

226   One day prior to the assay, 50,000 SW13 cells were seeded into a 96 well black-walled, clear

227   bottom tissue culture plate. All serum samples were heat inactivated at 56°C for 30 m. Half-log

228   serial dilutions were made in duplicate from 1:25 to 1:25,368 and then an equal volume of

229   medium with IbAr10200 VLPs containing 237 $TCID_{50}$ units was added and incubated at

230   37°C/5% CO_2 for 1 h. Final effective dilutions of analyte sera ranged from 1:50 to 1:50,736. Half

231   of this reaction mixture (50 µl) was then added to the previously aspirated target cell plate. Cells

232   were incubated for 24 h before being lysed using NanoGlo Lysis buffer mixed with 1/50 dilution

233   of NanoGlo substrate (Promega). Samples were mixed and incubated for 5 min at ambient

234   temperature prior to the luminescent signal being measured on a Modulus Microplate Reader

235   (Turner Biosystems) with an integration time of 5 s per well. To measure the effect of

236   complement on neutralization, Low-Tox Guinea Pig Complement (Cederlane Labs) was

237   reconstituted in DMEM, filtered, and added to the VLP/sera mixture at a final concentration of

238   5% and the assay was carried out as above. Data were analyzed as previously reported using

239   GraphPad Prism software (GraphPad Software) (33).

240   **CCHF$_{VLP}$ ELISA**

241   High Bind ELISA plates (Corning) were coated overnight at 4°C with approximately 1 ng N

242   equivalent of CCHF$_{VLP}$ diluted in PBS per 96-well plate. The following day, plates were washed

243   and then blocked with 3% goat serum/3% skim milk for 1 h at 37°C. All washes were done with

244   PBS containing 0.2% Tween-20 (PBST, Sigma). Plates were washed again, prior to being loaded

245   with two-fold serial dilutions of mouse sera in duplicate (dilution range 1:200 to 1:25,600).

246   Serum dilutions were carried out in blocking buffer. Plates were incubated at ambient

247   temperature for 1 h prior to being washed, and then incubated with a 1:1000 dilution of horse

248   radish peroxidase (HRP) conjugated goat anti-mouse (SeraCare Inc.) in PBST for 1 h at ambient

249   temperature. Plates were washed again and then developed with TMB substrate (SeraCare Inc.).

250   Absorbance at the 450 nm wavelength was detected with a Tecan M1000 microplate reader.

251   Pooled naïve sera collected prior to vaccination was used as an internal control on each assay

252   group. A plate cutoff value was determined based on the average absorbance of the naïve control

253   starting dilution plus 3 standard deviations. Only sample dilutions whose average was above this

254   cut-off were registered as positive signal. Additional analysis was carried out using GraphPad

255   Prism 6.

256   **Antibody isotype analysis**

257   Plates were coated with CCHF$_{VLP}$ as previously described. The following day, plates were

258   washed and blocked, and two-fold serial dilutions of mouse sera starting at 1:100 were added to

259   the wells of replicate plates. After 1 h incubation at ambient temperature, the plates were washed,

260   and then incubated for 1 h with a 1:10,000 dilution of either anti-mouse IgG1 HRP conjugated

261   antibody (Bethyl) or anti-mouse IgG2c HRP conjugated antibody (Bethyl). Plates were then

262   washed, and TMB substrate was added and absorbance at 450 nm absorbance was recorded.

263   **Antibody avidity**

264   Plates were coated with $CCHF_{VLP}$. The following day, plates were washed, blocked and loaded

265   with 1:200 dilutions of experimental sera for 1 h at ambient temperature. Plates were then

266   washed before being exposed to concentrations of Sodium Thiocyanate (Sigma-Aldrich) ranging

267   from 0-5.0 M. Samples were incubated for 15 m at ambient temperature before being washed.

268   Secondary antibody incubation and development with TMB substrate was then performed as

269   previously stated.

270   **Virus challenge and MAb-5A3 treatment**

271   C57BL/6 mice were treated by the intraperitoneal (IP) route with MAb-5A3 (Leinco

272   Technologies Inc) 24 h prior to (2.0 mg) and 24 h after (0.5mg) CCHFV challenge. IFNAR$^{-/-}$ and

273   IS C57BL/6 mice were challenged with 100 pfu of CCHFV strain IbAr 10200 by the IP route

274   four weeks following the final vaccination. The mice were monitored daily for weight change,

275   clinical score, and survival. Twenty-eight days following challenge the surviving mice were

276   euthanized by exsanguination under deep anesthesia, and sera were collected for post-challenge

277   analysis.

278   **N ELISA**

279   N antibodies in challenged mice were detected by ELISA. Recombinant N was produced as

280   previously reported (34) with minor modifications. Briefly, CCHFV N (strain IbAr10200) was

281   amplified and cloned into the vector pQE-30 (Qiagen) to have an N-terminal histidine tag. This

282   insert was transferred into the plasmid pFastbac-1 (Invitrogen) in order to generate recombinant

283   baculovirus according to manufacturer's instructions using the Bac-to-Bac Baculovirus

284   Expression System (Invitrogen). *Spodoptera frugiperda* (Sf9) insect cells were infected with the

285   recombinant baculovirus and incubated at 28°C for two days at 150 rpm. Cells were lysed (50

286   mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, 1% Tween20, pH 8.0), clarified, filtered

287   through a 0.45 µM filter, and loaded by syringe onto a nickel column (HisTrap FF, GE

288   Healthcare). The column was washed (50 mM NaH_2PO_4, 300 mM NaCl, 100 mM imidazole, pH

289   8.0), and the recombinant protein eluted (50 mM NaH_2PO_4, 300 mM NaCl, 150 mM imidazole,

290   pH 8.0). The elution buffer was exchanged with PBS though three successive concentrations

291   (10k YM centrifugal device, Millipore). Antigen concentration was determined using the

292   QuantiPro BCA Kit (Sigma-Aldrich).

293   For the ELISA, clear 96-well EIA (Corning) plates were coated overnight at 4°C with

294   100 µg of the purified N per well. The plates were then blocked in PBST with 3% nonfat dry

295   milk (BD Biosciences) and 3% goat serum (Corning) for 2 h at 37°C, and washed with PBST.

296   Twenty-eight day post-challenge, the sera were heat treated for 30 m at 56°C to inactive virus.

297   Sera were diluted in half-log dilutions in blocking buffer, starting at 1:50. The diluted sera were

298   added to the wells and the plates were incubated at 37°C for 1 h. The plates were then washed

299   with PBST, and probed for 1 h at 37°C with a 1:1000 dilution HRP-conjugated goat anti-mouse

300   antibody (Abcam), and then washed in PBST. The HRP was detected with TMB (SeraCare Inc.)

301   and the plates were read at 450 nm absorbance.

302   **Statistics**

303   Weight loss significance was determined using two-way ANOVA with the Bonferroni

304   correction. Survival statistics utilized the log-rank test**.** Statistical significance of $CCHF_{VLP}$

305   neutralization titers and ELISA data were assessed using the unpaired, two-tailed Student's *t* test

306   with log transformed titers. Significance levels were set at a p value less than 0.05. All analyzes

307   were performed using GraphPad Prism v.6.

308

Results

Expression of a gene-optimized CCHFV-M DNA vaccine construct

311   In earlier studies we found that a DNA vaccine expressing the M-segment ORF of CCHFV did

312   not consistently elicit neutralizing antibodies in vaccinated mice (23). In studies with a DNA

313   vaccine for Venezuelan equine encephalitis virus, we found that gene optimization could lead to

314   a dramatic improvement in expression and immunogenicity (30). Consequently, we generated a

315   new construct in which the CCHFV M-segment gene was optimized to reflect the codon bias of

316   humans and to remove known elements that impact mRNA stability and expression. Using flow

317   cytometry, we showed that a monoclonal antibody to CCHFV G_C detected viral protein both on

318   the surface of transfected non-permeabilized COS-7 cells and within permeabilized cells (Fig

319   1A). Expression levels were observed to be dose dependent (data not shown). Furthermore, we

320   confirmed expression of both CCHFV glycoprotein genes by Western blot using a rabbit

321   polyclonal antibody to detect G_N and a mouse monoclonal antibody to detect G_C (Fig 1B).

322

Fig 1. *In vitro* **expression of the glycoprotein genes from the CCHFV-M DNA vaccine**

plasmid. A. The total (permeabilized cells) and surface presence (non-permeabilized cells) of G_C

325   was examined 44 h after transfection of COS-7 cells with CCHFV-M or empty vector, 250 ng of

326   each plasmid. B. *In vitro* expression by Western blot of G_N (37 kDa) and G_C (75 kDa) in COS-7

327   cells 44 h after transfection of CCHFV-M or empty vector, 250 ng of each plasmid.

328

329 **Vaccination and challenge of IFNAR$^{-/-}$, and IS mice**

330 To compare the protective efficacy of the optimized CCHFV-M DNA vaccine in two lethal

331 mouse models, we vaccinated groups of 10 IFNAR$^{-/-}$ (C57/BL6 background) mice or

332 immunocompetent C57/BL6 mice three times at 3-week intervals by IM-EP with 25 µg of either

333 the CCHFV-M vaccine or empty pWRG7077 DNA plasmid vector. The mice were bled prior to

334 each vaccination to measure immune responses. Four weeks after the third vaccination, mice

335 were challenged by IP injection with 100 pfu of CCHFV. We chose the IP route for challenge, as

336 IP challenge of IFNAR$^{-/-}$ mice was previously found to result in a more rapid onset of disease

337 than challenge by subcutaneous, intranasal, or intramuscular routes (15); thus, the IP route

338 should provide a stringent test of the vaccine's efficacy. For the IS model, the vaccinated

339 C57/BL6 were immunosuppressed by treatment with an antibody to the IFN-α/β receptor 1 day

340 before and 1 day after challenge as described in Methods.

341 Following challenge, group weights (Fig 2A) and disease scores (not shown) were

342 obtained daily. All of the mice in both empty vector control groups displayed dramatic weight

343 loss and died or were euthanized between days 3 and 5 post-infection (Fig 2A, B). Both the

344 CCHFV-M vaccinated IFNAR$^{-/-}$ and IS mice lost between 5%-10% of their group weights by

345 day 6, but survivors returned to their starting weights by day 7 (Fig 2B) and had no visible signs

346 of illness (lethargy, ruffling). Three mice in the CCHFV-M DNA vaccinated IFNAR$^{-/-}$ group

347 died during manipulations three weeks following the final vaccination and prior to challenge.

348 Two out of seven (29%) CCHFV-M DNA-vaccinated IFNAR$^{-/-}$ mice died between days 4 and 5

349 post-infection, and four out of 10 (40%) CCHFV-M vaccinated C57/BL6 mice died or were

350 euthanized on day 5 post-infection. There was no significant difference between the survival

351   rates of CCHFV-M-vaccinated mice in the two mouse models, although there was a significant

352   difference in both models as compared to mice that were vaccinated with empty vector (Fig. 2B).

353

354   **Fig 2. The CCHFV-M DNA vaccination provided protection in both mouse models.** Weight

355   (A) and survival (B) following CCHFV challenge with 100 pfu by the IP route, vaccinated

356   C57BL/6 mice were transiently immunosuppressed prior to challenge (IS-B6). CCHFV-M

357   vaccinated group in each mouse strain compared to empty vector vaccinated group in the same

358   strain. Confidence intervals were set to 95%, *p=0.0002, **p<0.0001.

359

360   **Comparison of antibody responses of IFNAR$^{-/-}$ and immunocompetent C57BL/6 mice**

361   Total CCHFV glycoprotein-specific antibodies were measured after each vaccination by ELISA

362   using a CCHF$_{VLP}$ antigen. All of the CCHFV-M vaccinated mice developed CCHFV-specific

363   antibody responses following three vaccinations. The kinetics of the antibody responses were the

364   same for the immune competent C57/BL6 mice and the IFNAR$^{-/-}$ mice; i.e., both mouse strains

365   displayed detectable antibody responses after the first vaccination, large increases after the

366   second vaccination, and a smaller increase after the third vaccination (Fig 3A). Although

367   significantly higher total antibody titers were measured for individual IFNAR$^{-/-}$ mice vaccinated

368   with CCHFV-M as compared to C57/BL6 CCHFV-M vaccinated mice, there was no correlation

369   with ELISA titer and survival after challenge for either mouse strain (Fig 3B).

370

371   **Fig 3. CCHFV specific IgG ELISA titers increase following each vaccination.** The CCHFV

372   specific ELISA titers following three vaccinations are similar in IFNAR$^{-/-}$ and WT C578BL/6

373   mice before challenge. A) Mouse sera were pooled in each vaccination group and the CCHFV

374   specific IgG antibodies were measured by CCHF$_{VLP}$ ELISA following each vaccination with the

375   optimized CCHFV-M vaccine. Vaccinations were performed at weeks 0, 3, and 6. B). The

376   CCHFV IgG ELISA titers for individual mice, mice that died after CCHFV challenge are shown

377   in red. *Confidence intervals were set to 95%.

378

379   As an indirect measure of the Th1 vs Th2 response to the CCHFV-M DNA vaccine, we

380   performed IgG2c vs IgG1-specific ELISAs on samples collected 2 weeks after the final

381   vaccination. Both strains of mice had higher IgG2c then IgG1 responses (Fig 4A) indicating a

382   predominant Th1 response, which is consistent with previous trends seen in mice vaccinated by

383   IM-EP or needle delivery (30). All of the CCHFV-M vaccinated mice developed measureable

384   IgG2c responses and there was no significant difference between titers observed in the two

385   mouse strains. There was a significant difference in the IgG1 response between the IFNAR$^{-/-}$

386   group and the C57/BL6 group, with 42.8% (3 out of 7) of the IFNAR$^{-/-}$ mice having detectable

387   IgG1, and 80% (8 out of 10) of the WT C57/BL6 mice having detectable IgG1. The ratio of

388   IgG2c to IgG1 was greater in the IFNAR$^{-/-}$ mice than in the WT C57/BL6 (data not shown),

389   indicating that overall the immunocompetent mice may have a more balanced response than the

390   IFNAR$^{-/-}$ mice. We also confirmed the ability of our CCHFV-M DNA vaccine to induce affinity

391   matured B cell responses by avidity ELISA (Fig 4C). Estimated avidity of the CCHFV specific

392   antibodies in the CCHFV-M vaccinated IFNAR$^{-/-}$ group was significantly higher than the WT

393   C57BL/6 group, however, this did not correlate to a higher survival in the IFNAR$^{-/-}$ mice.

394

395

396   **Fig 4. The majority of the CCHFV-M vaccinated mice developed both IgG1 and**

397   **IgG2c responses following three vaccinations**. Prior to challenge, CCHFV-specific antibody

398   isotypes and the antibody avidity were examined by ELISA against the $CCHF_{VLP}$. A) The

399   CCHFV specific IgG1 and IgG2c response in individual mice following three vaccinations.

400   Pooled sera from $IFNAR^{-/-}$ and WT C57BL/6 mice vaccinated with empty vector were tested

401   concurrently and had no detectable signal (data not shown). B) The avidity of the CCHFV

402   specific antibody response in vaccinated mice following three vaccinations were measured. A)

403   and B) Mice that died after CCHFV challenge are shown in red. *Confidence intervals were set

404   to 95%.

405

406   To assess the neutralizing antibody responses to the CCHFV-M DNA vaccine we used a

407     $CCHF_{VLP}$ neutralization assay as described in the Methods. All of the CCHFV-M DNA-

408   vaccinated $IFNAR^{-/-}$ mice and 90% of the C57/BL6 mice developed neutralizing antibodies at

409   >1:50 dilution. Although there was no significant difference in the group titers of the two mouse

410   strains, the $IFNAR^{-/-}$ mice all had consistent antibody responses as compared to one another

411   whereas there was a wide range of responses among the C57/BL6 mice. There was no significant

412   difference in the CCHFV-specific neutralizing response between the survivors and the non-

413   survivors in either mouse model. For both mouse strains, the addition of complement

414   significantly increased the neutralizing antibody titers (Fig 5).

415

416 **Fig 5. CCHFV-M vaccinated mice developed CCHFV-specific neutralizing antibody**

417 **following three vaccinations, and the response was enhanced with the addition of**

418 **complement.** Prior to challenge, neutralization titers were measured against the CCHF$_{VLP}$, with

419 and without complement. Mice that died following challenge are highlighted in red. *Confidence

420 intervals were set to 95%.

421

422   Our CCHFV-M DNA vaccine was immunogenic in both mouse models, as all of the

423 vaccinated mice developed antibody responses to CCHFV. There was no clear correlation

424 between the humoral response to the vaccine and survival after CCHFV challenge in either

425 IFNAR$^{-/-}$ or WT C57/BL6 mice. Mice that developed a higher CCHFV-specific antibody

426 response, a higher IgG1 response, higher neutralizing antibody titers, or higher antibody avidity

427 did not have an increased chance of survival.

428

429 **Seroconversion of vaccinated mice following challenge**

430 To determine if mice that survived CCHFV challenge had overcome viral replication we

431 assessed antibodies to CCHFV N in sera collected from mice four weeks after challenge.

432 CCHFV N was not encoded in our vaccine construct, so the presence of anti-N antibodies would

433 suggest viral replication. All of the IFNAR-/- mice and all but one mouse of the IS C57BL/6

434 group had detectable antibodies to CCHFV N, indicating that the vaccine did not provide sterile

435 immunity (Fig 6). There was no difference in the N antibody response between the IFNAR$^{-/-}$ and

436 the IS mice.

437

438   **Fig 6. N-specific antibodies in post-challenge sera of CCHFV-M vaccinated mice.** Sera from

439   vaccinated mice that survived CCHFV challenge (28 days post-challenge) were examined by

440   ELISA for antibodies against N.

441

Discussion

443   We generated a DNA vaccine construct encoding the M-segment ORF of CCHFV, which was

444   optimized for expression in mammalian cells. We compared and quantified both the humoral

445   response and protective efficacy of our optimized DNA vaccine in two murine challenge models

446   (IFNAR$^{-/-}$ and IS) with the same genetic background (C57BL/6). We found that the optimized

447   CCHFV-M DNA vaccine delivered by IM-EP was highly immunogenic, with 100% of IFNAR$^{-/-}$

448   vaccinated mice and 90% of C57BL/6 mice developing CCHFV-specific immune responses,

449   including neutralizing antibodies. The single mouse in the IS model that failed to produce

450   neutralizing antibodies did develop G_N/G_C-specific antibodies, albeit at low levels. The

451   immunocompetent C57BL/6 mice developed a more balanced IgG2c/IgG1 response than the

452   IFNAR$^{-/-}$ mice, which may be due to cytokine signaling differences in the immunocompetent

453   mice. In both mouse models, the CCHFV-specific IgG ELISA titers of vaccinated mice

454   significantly increased between the second and third boosting vaccinations. Because we did not

455   test additional vaccinations, we do not know if we reached the maximum response possible.

456   As we were preparing this manuscript, another study reported DNA vaccination of

457   IFNAR$^{-/-}$ mice with an A129 background using separate plasmids expressing CCHFV G_N, G_C, or

458   N genes, each tethered to a ubiquitin coding sequence (22). In general, our findings of the

459   humoral immune response to DNA vaccination are in agreement with those in the other DNA

460   vaccine report; however, numerous differences between the two studies make it difficult to

461   directly compare results. For example, our vaccine expresses the complete M-segment ORF of

462   CCHFV, whereas in the other study a mixture of plasmids was used, which included an N

463   construct along with two constructs encoding the individual glycoprotein genes, from which the

464   mucin-like domain and GP38 coding regions were deleted. Also, our vaccine does not express

465   either the N gene or the ubiquitin sequence. The ubiquitin was intended to broaden the cell-

466   mediated immune response, but it is difficult to determine if it did due to the small number of

467   mice used and the limited sample volumes that precluded a comprehensive assessment of cell

468   mediated immunity. In addition to differences in the DNA vaccine constructs and the differing

469   genetic backgrounds of the $IFN^{-/-}$ mice, the studies differed in that we used a lower dose of DNA

470   and a different delivery method (IM-EP vs intradermal EP) and compared the $IFNAR^{-/-}$ mouse

471   model responses to antibody responses of immunocompetent mice

472   In our studies, we could not identify a correlate of protective immunity in either mouse

473   model. While we were able to elicit specific anti-CCHFV antibody responses in all vaccinated

474   mice, post-challenge seroconversion ELISA results revealed that the vaccine was not able to

475   prevent viral replication in the majority of the CCHFV-M DNA-vaccinated mice. These results

476   are similar to those reported previously with a MVA-GP vaccine, and in the recent CCHFV

477   DNA vaccine study (19, 22). Also consistent with the MVA-GP and the DNA vaccine studies,

478   we found no direct correlation between the humoral response(s) and survival in CCHFV-M

479   DNA-vaccinated mice (19, 21) suggesting that anti-CCHFV glycoprotein antibodies alone

480   elicited by these vaccines are not sufficient for protection to viral challenge. This is in agreement

481   with results of an earlier study demonstrating that the passive transfer of serum antibodies from

482   MVA-GP vaccinated mice into a naïve host did not confer protection to CCHFV challenge (21).

483 MVA-GP vaccine studies further suggested, through adoptive transfer of T-cells and passive sera

484 transfer studies, that both the cellular and humoral responses to the MVA-based CCHFV vaccine

485 were necessary to provide protection, as determined by a statistically significant delay in time to

486 death. Contrary to this, earlier passive transfer studies with monoclonal antibodies directed

487 against CCHFV G_N/G_C show that individual neutralizing and non-neutralizing antibodies alone

488 can provide 100% protection both before and after challenge in suckling mice (35). However,

489 monoclonal antibody studies have not been reported to confirm that this protection holds true in

490 an adult mouse model. As we have not yet assessed cell-mediated immune responses to our DNA

491 vaccine, we cannot eliminate the necessity for inclusion of additional immunogens, such as N, to

492 elicit T cell responses, although the immune response elicited by the MVA-GP vaccine was fully

493 protective, whereas MVA-N vaccine was unable to protect.

494 We show in this study that immune competent mice can be used to evaluate CCHFV

495 vaccines and protective efficacy can be examined by transient inhibition of IFN-I using MAB-

496 5A3 proximal to the time of challenge. IFN-α/β signaling is critical for the generation of potent

497 adaptive immune responses, for example by promoting antigen presenting cell maturation,

498 driving the T cell, and subsequent B cell, response (36, 37). IFN-α/β also amplifies B cell

499 receptor sensitivity, boosting the ability of naïve B cells to produce antibodies upon antigen

500 recognition (38). Furthermore, IFN-α/β signaling promotes the generation of memory T and B

501 cell pools. Although we did not observe significant differences either in antibody responses or

502 protective immunity in the IFNAR$^{-/-}$ vs the IS models with our CCHFV M DNA vaccine, the

503 ability to vaccinate immune intact mice might be advantageous for other DNA vaccine

504 approaches or for other types of CCHFV vaccines. For example, in the same study where the

505 mixed CCHFV DNA vaccine plasmids were tested, a transcriptionally-competent CCHF VLP

506   (tcVLP) vaccine was given alone or in a prime-boost regimen with the DNA. The IFNAR$^{-/-}$ mice

507   vaccinated with the tcVLP or the prime-boost were less protected than the DNA vaccine alone

508   (22). The authors concluded that a type I IFN responses may be required for the development of

509   a protective immune response against the tcVLP vaccine. Therefore, the ability to study CCHFV

510   vaccines in immune intact mice and then testing protective efficacy by disrupting IFN signaling

511   only at the time of challenge might have important advantages over the IFNAR$^{-/-}$ CCHFV

512   vaccination model, particularly when T cell responses are critical for protection (39, 40).

513   In summary, here we show that a novel CCHFV M-segment DNA vaccine can elicit

514   protective immune responses to CCHFV challenge in two lethal mouse models of CCHF. The

515   exact mechanism of protection remains unclear, but it is evident that a DNA vaccine encoding

516   the CCHFV M-segment ORF can generate protective immunity. It remains to be seen if this

517   vaccine can provide cross-protective immunity to more genetically distant CCHFV strains.

518   Overall, our results provide further insight into the protective capabilities of a CCHFV DNA

519   vaccine and will help in the development of a more rationally tailored CCHFV vaccine.
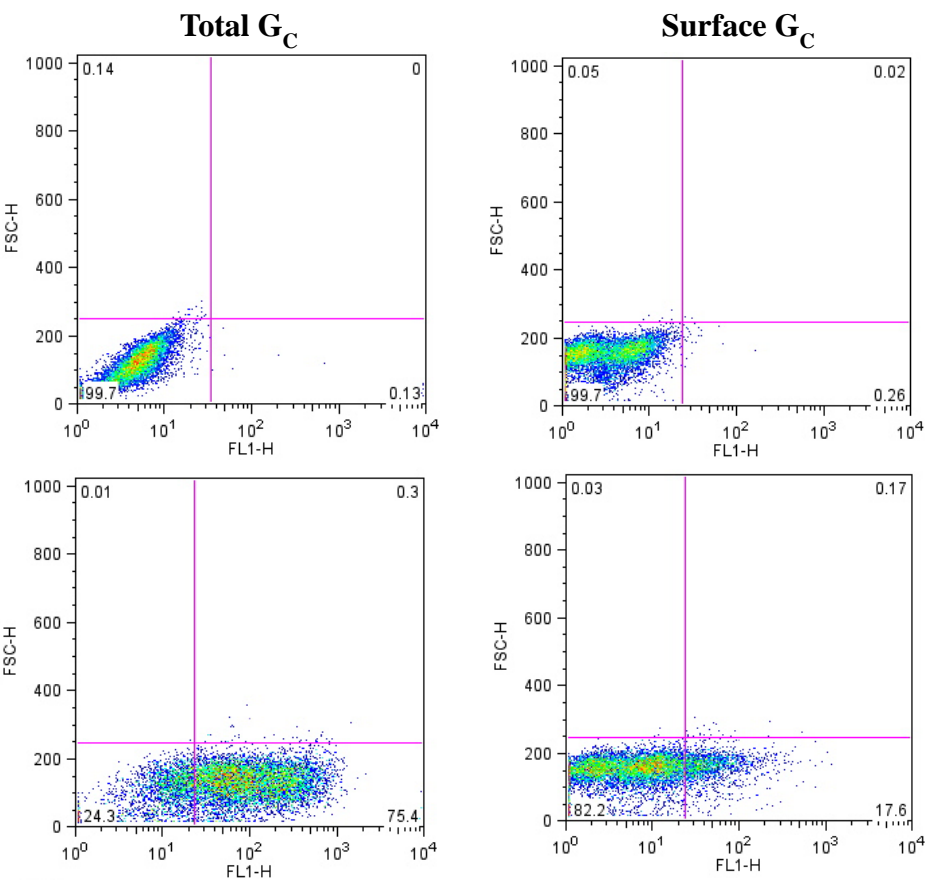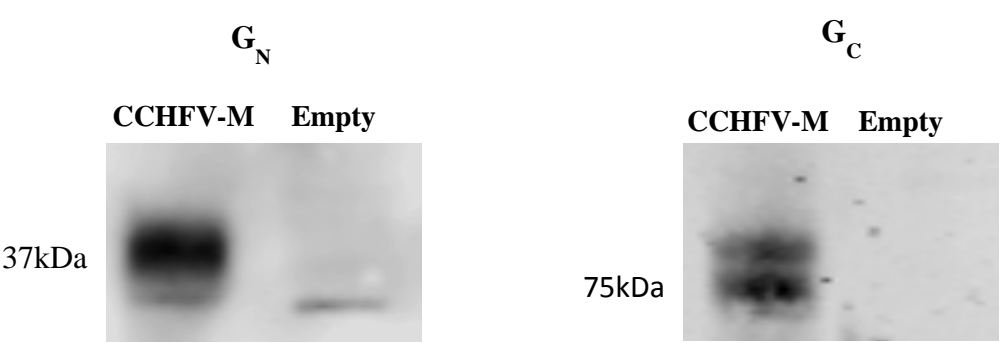
520

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A.

Fig 1. *In vitro* expression of the glycoprotein genes from the CCHFV-M DNA vaccine plasmid. A. The total (permeabilized cells) and surface presence (non-permeabilized cells) of G_C was examined 44 h after transfection of COS-7 cells with CCHFV-M or empty vector, 250 ng of each plasmid. B. *In vitro* expression by Western blot of G_N (37 kDa) and G_C (75 kDa) in COS-7 cells 44 h after transfection of CCHFV-M or empty vector, 250 ng of each plasmid.
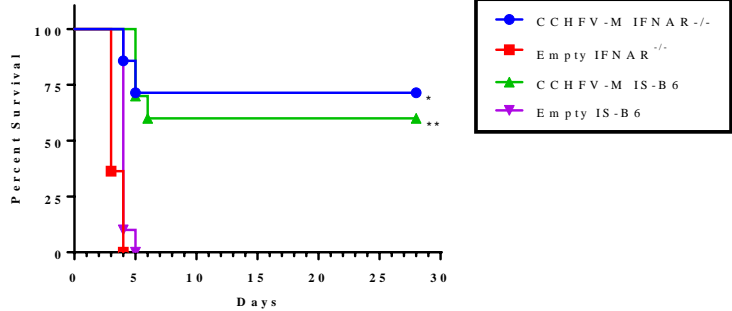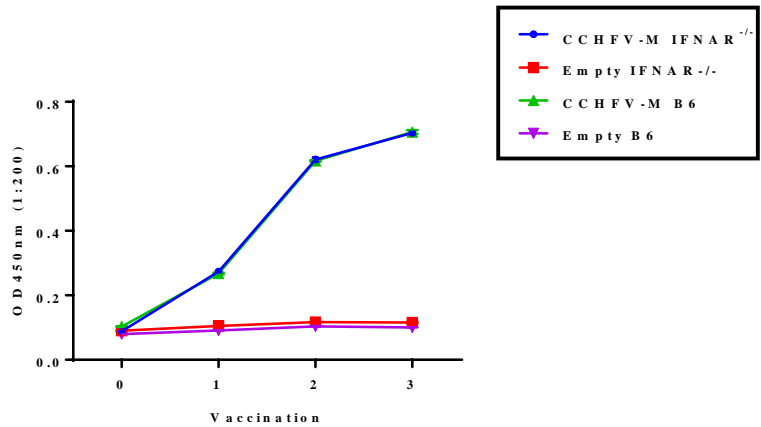
541 *A.*

542

543 *B.*

544

545 **Fig 2. The CCHFV-M DNA vaccination provided protection in both mouse models.** Weight

546 (A) and survival (B) following CCHFV challenge with 100 pfu by the IP route, vaccinated

547 C57BL/6 mice were transiently immunosuppressed prior to challenge (IS-B6). CCHFV-M

548 vaccinated group in each mouse strain compared to empty vector vaccinated group in the same

549 strain. Confidence intervals were set to 95%, *p=0.0002, **p<0.0001.

550

551

552 *A.*

553

554 *B.*

555

556 **Fig 3. CCHFV specific IgG ELISA titers increase following each vaccination.** The CCHFV

557 specific ELISA titers following three vaccinations are similar in IFNAR^{-/-} and WT C578BL/6

558 mice before challenge. A) Mouse sera were pooled in each vaccination group and the CCHFV

559 specific IgG antibodies were measured by $CCHF_{VLP}$ ELISA following each vaccination with the

560 optimized CCHFV-M vaccine. Vaccinations were performed at weeks 0, 3, and 6. B). The

561 CCHFV IgG ELISA titers for individual mice, mice that died after CCHFV challenge are shown

562 in red. *Confidence intervals were set to 95%.

563 *A.*

564

565 *B.*

566

567 **Fig 4. The majority of the CCHFV-M vaccinated mice developed both IgG1 and IgG2c**

568 **responses following three vaccinations**. Prior to challenge, CCHFV-specific antibody isotypes

569 and the antibody avidity were examined by ELISA against the $CCHF_{VLP}$. A) The CCHFV

570 specific IgG1 and IgG2c response in individual mice following three vaccinations. Pooled sera

571 from IFNAR^{-/-} and WT C57BL/6 mice vaccinated with empty vector were tested concurrently

572 and had no detectable signal (data not shown). B) The avidity of the CCHFV specific antibody

573 response in vaccinated mice following three vaccinations were measured. A) and B) Mice that

574 died after CCHFV challenge are shown in red. *Confidence intervals were set to 95%.
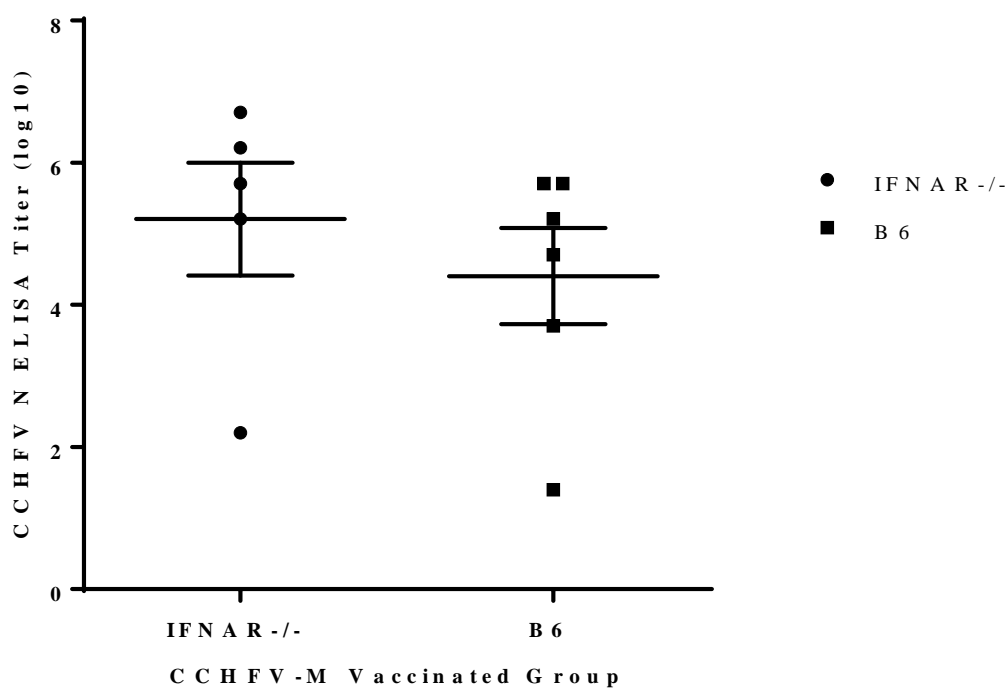
575

Fig 5. CCHFV-M vaccinated mice developed CCHFV-specific neutralizing antibody

following three vaccinations, and the response was enhanced with the addition of

complement. Prior to challenge, neutralization titers were measured against the $CCHF_{VLP}$, with

and without complement. Mice that died following challenge are highlighted in red. *Confidence

intervals were set to 95%.

581

582

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Fig 6. N-specific antibodies in post-challenge sera of CCHFV-M vaccinated mice. Sera from

vaccinated mice that survived CCHFV challenge (28 days post-challenge) were examined by

ELISA for antibodies against N.

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